Specification

Regenerating Dentin

Technical Field

The present invention relates to a regenerating dentin and also relates to a composite dentin material used for the regeneration. More specifically, the present invention relates to a composite dentin material containing noncollagenous phosphorylated protein and collagen and regenerating dentin using the composite dentin material.

Background Art

In recent years, artificial materials are frequently used for a transplantation to restore dentin in the dental field. For such artificial materials, it is required to having calcification inductivity - that is property absorbing the materials themselves while inducing a calcareous material after transplantation - in addition to biocompatibility as biomedical materials.

Although ceramics-based materials or the like are devised as materials for regenerating the dentin, a composite material of BMP (bone morphogenic protein) and collagen is believed to be more useful in calcification inductivity, and there is no other material which is expected sufficient calcification ability. However, although the BMP has strong calcification ability, the BMP is hardly soluble in water, has no optimum carrier found out, and is required needs a high concentration such as approximately 0.4 mg/ml for clinical application to human beings even though the BMP exhibits high calcification ability in small animals, like mice and rats. As a result, it is anticipated that the usage of BMP is limited to an expensive medical care in the present circumstances.

Therefore, it is desired to develop a safe and inexpensive bioabsorbable material having the calcification ability which replaces the BMP.

Although the inventors of the present application have already found out that phosphophoryn or phosvitin contained in the teeth independently has osteogenesis ability (Saito et al. Bone 21(4) 305-311(1997)), there is no report that these materials have actually been applied to dentin regeneration. Also, the inventors of the present application have reported a composite biomedical material for osteoanagenesis in which phosphophorin is cross-linked to collagen (JP, 2003-235953, A). However, it is impossible to anticipate an effect of regeneration of the dentin from the results of osteoanagenesis.

Materials for regenerating dentin are expected to be bioabsorbable and to have a mechanical property such as easily shaped and flexible in order to appropriately fill a site of periodontal disease or deficit of alveolar bone or dentin. In this relation, collagen type I is basically known as a main organic component of the alveolar bone and dentin, and known as a nucleous of calcification. Collagen type I can be prepared in various forms, such as a spongy form and a gel-like form. However, the calcification ability of collagen type I is extremely low to promote the dentin regeneration by itself.

Disclosure of the Invention

An objective of the present invention is to provide an inexpensive and safe for a regenerating dentin and a dentin material used for the regeneration.

As a result of keen examination to achieve the above objective, the inventors of the present application focus attention on the findings that the calcification ability is observed in noncollagenous phosphorylated protein, such as

phosphophoryn, phosvitin or DMP-1 (Dentin Matrix Protein-1). Then, the inventors of the present application figured out that an excellent dentin regeneration can be expected by differentiating dental pulp cells into odontoblasts on a scaffold of a composite material comprising the non-collagenous phosphorylated protein cross-linked to collagen.

The present invention relates to the as following. A dentin material is manufactured by seeding dental pulp cells in the composite material containing noncollagenous phosphorylated protein and collagen; and incubating the dental pulp cells.

A dentin material is manufactured by seeding dental pulp cells in the composite material containing noncollagenous phosphorylated protein and collagen; and incubating the dental pulp cells in the medium containing bone morphogenous protein.

A regenerating dentin placed in a cavity of dental pulp which is formed by seeding dental pulp cells in the composite material containing noncollagenous phosphorylated protein and collagen and incubating the dental pulp cells to differentiate into an odontoblasts and to proliferate; and implanting the odontoblasts into a cavity of dental pulp with said composite material.

A regenerating dentin placed in a cavity of dental pulp is formed by seeding dental pulp cells in the composite material containing noncollagenous phosphorylated protein and collagen; and incubating the dental pulp cells in the medium containing bone morphogenous protein to differentiate into a odontoblasts and to proliferate; and
implanting the odontoblasts into a cavity of dental pulp with said composite material.

The seeding density of the cells is 10^5 to 10^6 cells/ml.

A regenerating dentin placed in a cavity of dental pulp is formed by implanting the composite material containing

noncollagenous phosphorylated protein and collagen.

A regenerating dentin placed in a cavity of dental pulp is formed by implanting the composite material containing noncollagenous phosphorylated protein, collagen and bone morphogenic protein.

The rate of formation of regenerated dentin by the regenerating dentin material placed in a cavity of dental pulp is over 20% (after 2weeks).

The noncollagenous phosphorylated protein is phosphophoryn, phosvitin, DMP-1 or a mixture thereof.

The noncollagenous phosphorylated protein is chemically cross-linked with the collagen.

The composite material has spongy or gel-like structure which is manufactured by cross-linking the noncollagenous phosphorylated protein to the collagen by using divinyl sulfone or 1-ethyl-3-(3-dimethylaminopropyl)carbodiamide.

The composite material contains at least one or more materials selected from the group consisting of hydroxyapatite, β-TCP, α-TCP, polyglycolic acid, polylactic acid and a derivative thereof.

The collagen is collagen type I.

According to the present invention, it is possible to effectively regenerate defective dentin. The regenerating dentin and the dentin materials for dentin restoration of the present invention are inexpensive and safe, and therefore useful as a conservative treatment means in general dental care.

Brief Description of the Drawings

Fig. 1 shows a hematoxylin and eosin (HE) staining image photographed 1 week after pulp capping (transplantation) in rat.

Fig. 2 shows an HE staining image photographed 2 weeks

after pulp capping (transplantation) in rat.

Fig. 3 shows an HE staining image photographed 3 weeks after pulp capping (transplantation) in rat.

Fig. 4 shows an HE staining image photographed 2 weeks and 3 weeks with phosphophoryn/collagen/BMP after pulp capping (transplantation) in rat.

Fig. 5 shows an HE staining image photographed 3 weeks with phosphophoryn/collagen/BMP after pulp capping (transplantation) in dog.

In the above Figs. 1 to 3, the upper two staining images show the results of a phosphophoryn-collagen complex transplantation group (left: ×40; right: ×100), and the lower two staining images show the results of a collagen sponge transplantation group (left: ×40; right: ×100).
Symbols in the photographs indicate respectively D: dentin, P: dental pulp, and ND: new (regenerated) dentin.

Best Mode for Carrying out the Invention

The present invention relates to a regenerating dentin, characterized by incubating dental pulp cells on a scaffold of a composite material containing noncollagenous phosphorylated protein and collagen. The present invention is described in detail below.

1.Noncollagenous phosphorylated protein

As used herein "Noncollagenous phosphorylated protein" means phosphorylated protein having calcification ability other than the collagen. For example, phosphophoryn, phosvitin, DMP-1 (Dentin Matrix Protein-1), and the like, are mentioned.

(1) Preparation of phosphophoryn

Phosphophoryn is a phosphorylated protein contained in teeth, and known to have osteogenic ability by itself (J Biomater Sci Polymer Edn;1097-1103,2003). A phosphophoryn can be obtained as follows. The teeth (for example, bovines, pigs,

and the like) are extracted, and soft tissue, dental pulp, enamel, and cement are removed. Remaining dentin is pulverized finely, and the resultant material is decalcified by using an appropriate buffer solution (for example, 0.5 M EDTA, 0.05 M Tris-HCl, pH 7.4) containing protease inhibitors, dialyzed, and freeze-dried. Then, the obtained freeze-dried material is dissolved in a buffer solution (for example, 20 mM Tris-HCl, pH 7.4 [containing protease inhibitors]), and subsequently calcium chloride is added. Produced precipitates are dissolved in a buffer solution (for example, 0.5 M EDTA, 0.05 M Tris-HCl, pH 7.4 [containing protease inhibitors]), and the resultant solution is dialyzed and freeze-dried again. Finally, the resultant freeze-dried material is dissolved in a urea solution (for example, 4 M Urea, 0.01 M Tris-HCl, pH 8.0), and then phosphophoryn is separated by using ion-exchange chromatography (for example, DEAE-Sepharose, or the like) or the like. The objective phosphophoryn can be identified by phosphoric acid analysis and amino acid analysis.

(2) Phosvitin

Phosvitin is a main component of vertebrate yolk protein, and is a phosphorylated protein contained in yolk granules. Hen's egg phosvitin has a molecular weight of approximately 100,000 and contains approximately 10% phosphoric acid, wherein approximately half of amino acids are serine and most of the serine residues are phosphoserine residues. The phosvitin is known to have the calcification ability by itself similar to the phosphophoryn (Bone. 1997 Oct; 21(4): 305-11). As the phosvitin, commercially available products (Sigma Chem. Co., or the like) can be used, and it can be prepared easily according to the previous report (Shainkin R, Perlmann GE., "Phosvitin, a phosphoglycoprotein. I. Isolation and characterization of a glycopeptide from phosvitin." J Biol Chem. 1971 Apr 10; 246(7): 2278-84.).

(3) DMP-1 (Dentin Matrix Protein-1)

DMP-1 is secretory noncollagenous acidic phosphorylated protein identified from a dentin cDNA library, and participates in calcification in extra-cellular matrices such as bone and dentin (George A. et al. and J Biol Chem. 1993 Jun 15; 268(17): 12624-30.). A DMP-1 gene is located at 4q21 in the human chromosome, and forms a family with osteopontin gene, MEPE gene, bone sialoprotein gene, and the like which are located in the vicinity. Since the DMP-1 becomes negatively charged in tissue because of its amino acid sequence and is combined with a calcium ion, the DMP-1 is considered to be closely related to the calcification. It is actually reported that in experiments on DMP-1 gene introduction to osteoblastic cell lines, promotion of calcification was observed (Feng JQ, et al., and J Dent Res. 2003 Oct; 82(10): 776-80), and that the deficiency of the DMP-1 gene causes the formation inhibition of teeth accompanied by abnormality in dentin formation or hypocalcification (Ye L. et al. J Biol Chem. (2004) Feb. 13 [Epub ahead of print]). As described above, the DMP-1 is considered to be an important molecule for the calcification of bone or teeth (in particular for dentin formation).

DMP-1 used in the present invention can be prepared by gene engineering, or extraction and purification from the dentin of bone or teeth according to a well-known method (George A. et al., J Biol Chem. 1993 Jun 15; 268(17): 12624-30.).

2. Collagen

As collagen, in the present invention, collagen type I which accounts for most of the organic matters of a bone or a tooth and has high biocompatibility is preferably used. The collagen type I is commercially available and may be prepared in accordance with the publicly known method. For example,

collagen is extracted from an appropriate material (e.g. connective tissue of animals such as the skin of bovines or pigs) and purified in accordance with the publicly known method. A reconstituted collagen type I fiber can be obtained by freeze-drying and dissolving the purified collagen in an acetic acid solution, adding NaCl, NaOH, Hepes, and the like, and incubating the mixture.

3. Composite material consisting of noncollagenous phosphorylated protein and collagen

A composite material of the present invention contains noncollagenous phosphorylated protein and collagen. In the composite material, the blend ratio (mass ratio) of the noncollagenous phosphorylated protein to the collagen is preferably 1:10 to 1:50, and more preferably 1:20 to 1:40. The noncollagenous phosphorylated protein is preferably blended by 2 to 10 mass % (hereinafter, "mass %" is simply called %) to the total amount (total mass) of the composite material, and more preferably by 2.5 to 5.0%. The composite material which contains low noncollagenous phosphorylated protein has insufficient calcification ability. On the other hand, the composite material which contains much noncollagenous phosphorylated protein costs a lot of money.

In the above composite material, the noncollagenous phosphorylated protein is preferably chemically cross-linked to collagen fibers. As a cross linking agent, divinyl sulfone, 1-ethyl-3-(3-dimethylaminopropyl)carbodiamide, or the like, can be used.

For example, first, the collagen fibers are dissolved in an aqueous solution of a carbonate, such as potassium carbonate and sodium carbonate, and incubated at room temperature. The concentration of the carbonate aqueous solution is preferably 0.4 M to 0.5 M, and more preferably 0.1 M to 0.2 M. The cross linking agent, for example, divinyl

sulfone, 1-ethyl-3-(3-dimethylaminopropyl)carbodiamide, or the like, is added to the solution to introduce a cross-linking chain on the collagen fibers in advance. In the case of divinyl sulfone, the amount of the cross linking agent to be added is preferably approximately 5.0 mass %.

Next, the noncollagenous phosphorylated protein is added and incubated to be cross-linked with the collagen. The amount of noncollagenous phosphorylated protein to be added is preferably 1/10 to 1/50 of the collagen, and more preferably 1/20 to 1/40 of the collagen (mass ratio). The resultant material is washed with distilled water, and then with a bicarbonate (for example, sodium bicarbonate, potassium bicarbonate, or the like) solution to remove surplus noncollagenous phosphorylated protein and cross linking agent. Finally, sodium bicarbonate and mercaptoethanol are added to stop the cross-linking reaction, and the resultant material is thoroughly washed with distilled water.

The composite material after cross-linking may be directly heated to be gelated and used as a gel-like structure, or may be freeze-dried to be used as a spongy structure. This gel-like or spongy structure provides characteristics suitable for dental pulp cell incubation or dental deficient part supplementation to be described.

When producing the spongy structure, the conditions of the freeze-drying (for example, temperature, freezing time, underwater freeze-drying, and the like) can be suitably adjusted according to the structure of a desired composite material, that is, a specific surface area, porosity, the size of pores (voids), and the like. Further, the obtained freeze-dried material can be molded if needed and for example utilized as a dental implant or the like to be described. The "spongy structure" shall mean a minute porous structure (structure in which numerous pores (voids) of approximately

several micrometers to several tens of micrometers exist) having flexibility. In the composite material having the spongy structure of the present invention, the porosity is preferably 40 to 90%, and more preferably 60 to 90%. With porosity exceeds the above range, cell penetration is insufficient to deteriorate calcification ability, and the strength of the composite material itself is diluted.

In addition to the indispensable components of noncollagenous phosphorylated protein and the collagen, the composite material having the spongy structure may contain a porous hard material, such as hydroxyapatite, βTCP, αTCP, polyglycolic acid, polylactic acid, or its derivative (for example, a polymer of PLLA (poly-l-lactic acid) and PDLA (poly-d-lactic acid)), and the like, within the scope without ruinning the objective and effect of the present invention.

4. Composite dentin material consisting of noncollagenous phosphorylated protein and collagen and BMP.

Bone Morphogenetic Protein (BMP) is differentiation growth factor which belongs to Transforming Growth factor-b super family, and is known to induce a differentiation from undifferentiated mesenchymal cell to osteoblast and to induce osteogenesis. In vivo, inplantation of BMP with noncollagenous phosphorylated protein and collagen into subcutaneous or defective part of bone induces enormous osteogenesis. The BMP used in the present invention is recombinant human BMP-2 variant.

5. Regeneration of dentin

Dental pulp cells can be differentiated effectively into odontoblasts by seeding dental pulp cells on a scaffold of the composite material of the present invention and incubated under suitable conditions. As dental pulp cells, the dental pulp cells isolated from a patient himself/herself can be used suitably.

The cells may be merely seeded on a scaffold of the composite material or seeded after mixing the cells with a liquid, such as a buffer solution, physiological saline, a solvent for injection and a collagen solution. In the case that the composite materials do not allow smooth introduction of the cells into the pores of the composite materials, seeding may be performed under a suction condition. The number of cells to be seeded (seeding density) is preferably adjusted for more effective regeneration of the dentin. The seeding density of the cells is 10^5 to 10^6 cells/ml.

As a culture medium used for cell culture, a publicly known culture medium, such as a MEM culture medium, an α-MEM culture medium, and a DMEM culture medium, can be suitably selected according to a cell and used. Further, an antibiotic, an antimicrobial agent, a growth factor, a transcriptional factor, or the like such as FBS (manufactured by Sigma Chem. Co.) and Antibiotic-Antimycotic (manufactured by GIBCO BRL) may be added to the culture medium. It is preferred in particular to add bone morphogenetic protein (BMP), which is a growth factor to promote calcification. The culture is usually carried out under the conditions of 3 to 10% CO_2 and 30 to 40°C, in particular under the conditions of 5% CO_2 and 37°C, but not limited to these conditions. A culture period is preferably at least 3 days or more, but not limited to this period, and is determined according to circumstances.

Thus, the odontoblasts differentiated and derived from the dental pulp cells are further proliferated, and implanted or injected into the defective part with a scaffold of the composite material, and then the tooth dentin can be effectively regenerated.

A carious tooth is removed by cutting under infiltration anesthesia. In the case that dental caries is deep enough to cause pulp exposure, the tooth is washed with 8% NaClO and

with 3% H_2O_2 alternately, and then washed with a physiological saline solution and stop bleeding by applying pressure with sterilized cotton ball and dried. Then the tooth is attached a composite material of phosphophoryn/collagen or a BMP added composite material of phosphophoryn/collagen and is sealed by glass ionomer cement and composite resin.

6. Composite dentin material for dentin restoration (prosthetic implant)

The composite material obtained according to the present invention exhibits spongy elasticity by absorbing water, and has excellent biocompatibility and calcification ability. Further, since the composite material is easily molded and has a flexible mechanical property, it can appropriately fill a small space such as a dental defective part. That is, when the composite material of the present invention is implanted into the dental defective part, the composite material is promptly combined with surrounding tissue, and the interface between donor side tissue and the composite material can be integrated completely. Therefore, the composite material of the present invention itself can be used as an implant for restoring and regenerating the dentinal defective part.

When used as the implant, the composite material may have the spongy structure or the gel-like structure. The shape of sponge and the hardness of gel is freely adjustable according to defective parts to be filled or operability. Further, other physiologically active substances, medicines, or the like can be impregnated into the composite material. For example, if an anti-inflammatory agent is impregnated and released gradually, the postoperative inflammation of a dental pulp defective part can be prevented effectively.

Regarding the implant for dentin restoration, dental pulp cells may be seeded into the composite material and differentiated into odontoblasts and proliferated in vitro,

and then implanted in the defective part with a scaffold of the composite material. As dental pulp cells, cells derived from a patient can be used for more ideal implant for dentin restoration.

Example

In the following, the present invention is described in more detail with a reference example and a test example. The present invention is not limited to these examples.

[Reference example]

Manufacturing of composite material of phosphophoryn and collagen

(1) Purification of phosphophoryn

First, a permanent tooth is extracted from a bovine jawbone, and the soft tissue, the dental pulp, the enamel, and the cement are removed. Remaining dentin is pulverized to fine grains of 200 meshes or smaller in liquid nitrogen. The dentin powder is decalcified by using 0.5 M EDTA and 0.05 M Tris-HCl, pH 7.4 [containing protease: 100 mM 6-aminohexanoic acid (manufactured by Wako Pure Chemical Industries, Ltd.), 5 mM benzamidine-HCl and 1 mM phenylmethylsulfonyl fluoride] at 4°C.

Then, the EDTA decalcification solution is dialyzed against deionized distilled water by using a dialysis membrane (SPECTRUM MWCO 3500, 132725) at 4°C, and is subjected to freeze-drying (a product manufactured by Tokyo Rika Kikai: EYELA FREEZ DRYER 90500042). The resultant EDTA extract is dissolved into 20 mM Tris-HCl, pH 7.4 (containing protease inhibitor), and $CaCl_2$ is added so that a final concentration becomes 1 M. Precipitates are recovered by centrifugal separation (manufactured by Hitachi Koki: HIMAC CENTRIFUGE345043), and dissolved in 0.5M EDTA, 0.05 M Tris-HCl, pH 7.4 (containing protease inhibitor) again. The resultant solution is then dialyzed against deionized distilled water

and subjected to freeze-drying. The freeze-dried material is dissolved in 4 M Urea, 0.01 M Tris-HCl, pH 8.0, and eluted by using DEAE-Sepharose (manufactured by Sigma Chem. Co.) Column Chromatography according to 0 to 1 M NaCl linear gradient.

Finally, the phosphophoryn is identified by phosphoric acid analysis and amino acid analysis.

(2) Purification of collagen type I

The bovine skin is shredded and washed at 4°C with distilled water, 20% NaCl, 0.05 M Tris-HCl, pH 7.4. Extraction is performed overnight in 1 M NaCl, and 0.05 M Tris-HCl, pH 7.4. Then supernatant is recovered by centrifugal separation, and 0.5 M acetic acid and 1 M NaCl are added to the supernatant and the resultant mixture is stirred overnight.

Residue is recovered by centrifugal separation, dissolved in 0.5 M acetic acid, and centrifugal separation is further carried out. Supernatant is neutralized by using 5 M NaOH and 4.4 M NaCl. The resultant solution is stirred overnight, and centrifugally separated. To the residue, 4.4 M NaCl, 0.05 M Tris-HCl, pH 7.4 is added. The resulting mixture is stirred overnight, and then subjected to centrifugal separation.

To the residue, 2.4 M NaCl, 0.05 M Tris-HCl, pH 7.4 is added. The resultant mixture is stirred overnight, and then subjected to centrifugal separation. To the residue, 1.7 M NaCl, 0.05 M Tris-HCl, pH 7.4 is added. The resultant mixture is stirred overnight, and then subjected to centrifugal separation. The obtained supernatant is dialyzed against 0.1 M acetic acid, and is subjected to freeze-drying.

A 0.3% collagen acetic acid solution is prepared by using the resultant freeze-dried collagen and 50 mM acetic acid. Reconstituted collagen type I fibers are obtained by adding 0.15 M NaCl, then 0.6N NaOH and 0.1 M Hepes (manufactured by Wako Pure Chemical Industries, Ltd.), and

incubating the resultant mixture at 37°C.

(3) Cross-linking of phosphophoryn with collagen

The collagen fibers obtained in (2) is incubated in 0.5 M sodium carbonate at room temperature overnight. Further, divinyl sulfone (manufactured by Sigma Chem. Co.) is added, and the resultant mixture is incubated for 2 hours. After washing the collagen fibers thoroughly with 0.5 M sodium carbonate, the phosphophoryn is added and cross-linked by incubating overnight. The resultant material is washed with distilled water, and then thoroughly washed with 0.5 M sodium bicarbonate to remove excess phosphophoryn and divinyl sulfone.

Then, the crosslinking reaction is stopped by adding 0.5 M sodium bicarbonate and mercaptoethanol and incubating overnight. The obtained complex is washed with distilled water, and further with 0.5 M NaCl, 0.05 M Tris-HCl, pH 7.4, and distilled water.

The complex obtained by the above processes were freeze-dried, and thus the collagen-phosphophoryn complex (spongy sheet) was manufactured. Further, only collagen fibers to which no phosphophoryn is added were thoroughly washed with distilled water, and freeze-dried to manufacture a collagen complex (spongy sheet) which does not contain the phosphophoryn.

[Example 1]

Dentin regeneration test by using rats

1.Sample pulp capping

A male Wister rat (8 weeks, about 250g body weight) is kept the mouth opening by using mouth opener under intraperitoneal general anesthesia (1.6ml/ml). The intra oral area is disinfected with oxydol. Artificial pulp exposure is formed at mesial first premolar of upper jaw with sterilized diamond points (40ss-s, Matsubara, Kyoto) and sterilized round

bar (O14, Dentsply, tokyo). In order to stop bleeding at pulp exposure surface and to deinfect the cavity, the tooth is washed with 10% NaOCl and with 3% H_2O_2 alternately. Then the pulp expusure is covered with a composite material of phosphophoryn/collagen or a BMP added composite material of phosphophoryn/collagen.

The tooth is double coverd by GC (HY-BOND GLASONOMER CX, Matsukaze), Tri-S bond (kuraray medical, Okayama) and CR (UnifilLoFlo, GC, Tokyo).

Then remove dental antagonist and finished.

At the time the experience period has exhausted, carry out anesthetic death with diethyl ether and extirpate the tooth together with upper jaw by using surgical knife and gum scissors.

2. Sample extraction

The samples after transplantation were extracted from the rats in accordance with the following protocol.

1) The rats were subjected to anesthetic death by using triple doses of Ravonal (thiopental sodium).

2) Immediately after the extraction of the test teeth by using a dental elevator and forceps, the test teeth are fixed in a neutral formalin solution.

[Example 2]

Dentin regeneration test by using dogs

1. Sample pulp capping

Dental pulp defective parts of dogs (each N = 3) prepared experimentally in accordance with the following protocol were filled with a composite material of phosphophoryn/collagen or a BMP added composite material of phosphophoryn/collagen, collagen spongy as a control, and calcium hydrate which is historically used. And an effect of regenerating dentin was confirmed.

1) Atropine sulfate 2 ml, intramuscular injection

2) Horizon (diazepam preparation)
4 ml, intramuscular injection

3) Ketalar (ketamine hydrochloride)
10 ml, intramuscular injection

4) Legs are shaved by using a hair clipper for vein securement.

5) Venous paths are secured and fixed by using a butterfly needle.

6) Ravonal (thiopental sodium) 2 to 3 ml is administered. (When rats begin to awake during the operation, 1 to 2 ml is to be adminisered)

7) Potacol (containing Viccillin 250 mg) (electrolyte) is intravenously dripped during the operation.

Alternatively, Viccillin (ampicillin sodium) is added to a physiological saline solution, and the solution is intravenously dripped.

8) Local anesthesia is applied to test tooth sites by using 2% xylocaine (containing lidocaine hydrochloride epinephrine).

9) Cavity preparation

Test teeth

Upper jaw: Incisors Nos. 2 and 3, dogteeth, premolars Nos. 2 and 3

Lower jaw: Incisors No. 3, dogteeth, premolars Nos. 2 and 3

A cavity of approximately 2 to 3 mm in diameter is formed in the test teeth by using an air turbine diamond point. An exposure pulp surface of approximately 1 mm is formed by using a 2 mm steel round bur under irrigation with physiological saline.

10) Washing and the arrest of hemorrhage.

11) Each sample (composite material of phosphophoryn/collagen or a BMP added composite material of phosphophoryn/collagen, collagen spongy as a control, and calcium hydrate) was put on

the cavity surface.

12) Temporary sealed by using glass ionomer cement.

13) Further, restoration is carried out by using a composite resin.

14) A Voltaren suppository (diclofenac sodium) is administered for preventing inflammation, and then the operation is completed.

Results

The extracted samples (phosphophoryn-collagen complex and collagen sponge) were subjected to hematoxylin and eosin staining (HE staining), and histologically observed by using an optical microscope. The dentin regeneration effect was evaluated by further morphometry of the dentin.

(1) rat example

When the phosphophoryn-collagen complex was transplanted, extremely rapid (two weeks) dentin regeneration was observed compared to the collagen sponge (control). After three weeks of transplantation, vigorous dentin regeneration was observed in the phosphophorin-collagen complex group. On the other hand, in the collagen sponge group, the regeneration of the dentin was scarcely observed for three weeks.

Although the above reference example and the test example for an experimental protocol for the phosphophoryn collagen complex, the dentin regeneration effect can be confirmed similarly by preparing a phosvitin-collagen complex and a DMP-1-collagen complex.

Usage of a composite material of phosphophoryn/collagen and BMP formed more dentin at higher density than usage of a composite material of phosphophoryn/collagen for all experimental period (3 weeks). Futhermore, the usage of composite material of phosphophoryn/collagen/BMP has brilliant sealing ability and caused less inflammatory reaction. Calcium

hydrate which is historically used had no advantage over both of the composite material in all of forming volume of dentin, density of the dentin, sealing ability and biocompatibility. Only collagen or a composite material of collagen/BMP result about the same as calcium hydrate which is historically used (see the table 1).

(2) dog example

Two month after implantation, usage of a composite material of phosphophoryn/collagen/BMP formed more dentin than usage of a composite material of phosphophoryn/collagen, however usage of both of the composite materials result 100% of the density of the formed dentin. As for sealing ability, a composite material of phosphophoryn/collagen/BMP is exellent. Calcium hydrate preparation had no advantage over a composite material of phosphophoryn/collagen/BMP and a composite material of phosphophoryn/collagen in forming volume of dentin, density of the dentin, sealing ability and biocompatibility (see the table1).

Industrial Applicability

The regenerating dentin and the dentin material for dentin restoration of the present invention are inexpensive and safe, and can be used as a conservative treatment method in general dental care which is not limited to expensive medical care.

Table 1

Sample	Material	Extirpation time	Forming ability of dentin(%)	Density(%)	Sealing ability	inflammation
Rat	PP pcol-BMP	1w	5.2	89	D	B
		2w	30.3	93	B	B
		3w	54.8	99	B	A
	PP pcol	1w	3.5	74	D	B
		2w	22.9	95	D	B
		3w	43.3	98	B	B
	Ca	1w	1.6	68	C	C
		2w	16.2	76	C	C
		3w	39.2	88	B	B
	col-BMP	1w	2.3	79	D	C
		2w	16	90	C	B
		3w	32.1	95	B	B
	col	1w	2.1	63	D	C
		2w	14.5	88	C	B
		3w	29.2	90	C	B
Beagle	PP pcol-BMP	2w	48.1	100	A	B
	PP col	2w	14.4	100	C	B
	Ca	2w	9.1	100	C	B

Forming ability of dentin(%);Forming volume of dentin at cavity of dental pulp

Density(%);Total density of formed dentin

Sealing ability

A; Almost completely sealed

B; Approximately sealed but remain some cavity

C; dentin-like but not sealed

D; Dotted and not sealed

E; No dentin formation

Degrees of inflammation

A; No inflammation

B; Low-grade inflammation(slight hyperemia)

C; High-grade inflammation(infiltration of inflammatory cells)

D; Degeneration of dental pulp(vacuole degeneration)

E; Necrosis